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Prognosis in African-American Women

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# REPORT

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13. ABSTRACT (Maximum 200 Words)  Breast Cancer (BC) is the second leading cause of death among women in the United States. Although the incidence of breast cancer is higher in American White (AW) women, mortality in African American (AA) is considerably higher. These differences are perhaps due to histological and socioeconomic factors. Mutations of the tumor suppressor gene p53 are among the most common genetic defects in cancer cells, and in several studies alterations in p53 in breast cancer have been associated with a poor prognosis. Individual carrying mutations in p53 or inactivation of BRCA1 genes are predisposed to a variety of cancers, both tumor suppressor genes have been implicated in establishing genome stability by participating in DNA damage pathways. There have been discrete p53 mutations in AA cohort, which were different WA cohorts. Mutations in BRCA1 gene accounts for about 50% of inherited breast cancer cases, but somatic mutations of BRCA1 gene are absent in sporadic cancers. Inactivation of BRCA1 occurs via the hypermethylation of the promoter region of the BRCA1 gene in sporadic cancers. We have compared the the mutation of p53 gene and inactivation of BRCA1 gene in AA and AW population. We found that: 1)Higher p53 overexpression, representative of presence of mutant p53 protein, was observed in AAs then in AWs woman; 2) The number of p53 mutations were more in AA as compared to AWs; 3)Hypermethylation of promoter of BRCA1 gene was seen in cases where p53 was muted irrespective of race. This study, when complete will establish a causal variation in AAs as compared to WAs.				
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**Abstract:** Breast Cancer (BC) is the second leading cause of death among women in the United States. While the incidence of breast cancer is higher among American White (AW) women, than among African American (AA) women, mortality is considerably higher in the latter. These differences are likely due to both genetic and socioeconomic factors. Mutations of the tumor suppressor gene p53 are among the most common genetic defects in cancer cells, and several studies have identified alterations of p53 in breast cancer as indication of a poor prognosis. Individuals carrying mutations in p53 or inactivation of BRCA1 genes are predisposed to a variety of cancers; both tumor suppressor genes have been implicated in establishing genome stability by participating in DNA damage repair pathways. p53 mutations in the AA cohort, have been found to be distinctly different from those in WA cohorts. Mutations in the BRCA1 gene appears in about 50% of inherited breast cancer cases, but somatic mutations of the BRCA1 gene are absent in sporadic cancers. Inactivation of BRCA1 occurs via the hypermethylation of its promoter region in sporadic cancers. We have compared the status of abnormal p53 and inactivation of the BRCA1 gene in AA and AW women. We found: 1) higher p53 overexpression, characteristic of the presence of a mutant p53 protein, in AA than in AW women; 2) the number of p53 mutations were greater in AA than in AW women; 3) an inverse relationship ( $r = -0.73$ ) between premenopausal age and status of p53 was evident only in AA women and not in AW; 4) hypermethylation of the promoter of the BRCA1 gene was seen in cases where p53 was muted irrespective of race. This study points to a variation in the tumor suppressor p53 that may underly the differences in cancer mortality between AA and AW women. In addition, this study shows that loss of BRCA1, with or without p53 abnormalities, is not an independent prognostic marker in AA women, but rather a biological phenomenon associated with the global hypermethylation pattern.

## INTRODUCTION

Breast Cancer (BC) is the second leading cause of death from cancer among women in the United States. Although the incidence of breast cancer is 13% higher in American White (AW) women than in African Americans (AA), the latter have 28% higher mortality from breast cancer (Harris et al., 2003). These differences are perhaps due to both genetic and socioeconomic factors. Mutations of the tumor suppressor gene p53 are among the most common genetic defects in cancer cells. In several studies, alterations in p53 in breast cancer have been associated with a poor prognosis. Overexpression of p53 protein occurs with similar frequency in breast cancers in AA as well as in AW women (Rose and Royak-Schaler, 2001). Studies have also suggested that AAs have a different spectrum of p53 mutations compared to WAs, yet the specific mutational differences between these studies are somewhat conflicting (Blaszyk et al., 1994; Shiao et al., 1995). In addition, AAs with p53 mutations had definitely much poorer prognosis than did AWs (Shiao et al., 1995). The available reports in the literature, thus, provide several potential biological reasons for the poorer prognosis for breast cancer in AA women. Although, germline alterations in the BRCA1 gene are responsible for 50% of familial breast cancer (Friedman et al., 1994; Futreal et al., 1994), the mutation in the BRCA1 gene is not found to be mutated in sporadic breast cancer (Marajver et al., 1995; Berchuck et al., 1998). Recent studies have demonstrated that inactivation of BRCA1 in sporadic cancer can occur due to hypermethylation of its promoter region. (Esteller et al., 2000; Hedenfalk et al., 2001). This may symbolize a mechanism by which the BRCA1 gene gets inactivated in some sporadic cancers. Furthermore, loss of the tumor suppressor gene BRCA1 in sporadic forms of breast cancer may point to a novel mechanism with regard to its role in tumor initiation. Despite this fact, there have been no published studies determining whether breast cancers arising in AA women have an increased frequency of defects in either the p53 or BRCA1 gene or both, and whether there are specific types of defects associated with the two genes that distinguish these tumor groups. Mutations in p53 and BRCA1 have been studied; however, the possible relationship of p53 and BRCA1 hypermethylation has not been defined.

The main objective of this research project was to determine whether the alterations in p53 and BRCA1 genes that are involved in tumor development and progression are distinctly different in the AA population as compared to AW. We proposed to analyze the mutations of the tumor suppressor gene p53 that are among the most common genetic defects in cancers, and we planned to study the mutational spectrum of p53 in both population. Furthermore, we planned to establish that in addition to abnormal p53, inactivation of the BRCA1 gene via hypermethylation of its promoter, may contribute specifically to breast cancer in AA.

### Hypothesis/Rationale/Purpose

Overexpression of p53 protein has been observed at similar frequency in breast tumors from AW and AA women. Limited published data suggest that tumors from the AA group may exhibit a different spectrum of p53 mutations (Blaszyk et al., 1994; Shiao et al., 1995). In addition, BRCA1 gene inactivation may go along with p53 mutation and may contribute to greater virility of the cancer development, which is more aggressive in AA women. Therefore, we assessed sporadic breast cancers from AA and AW women for the following:

- 1) Overexpression of p53 protein and/or sequestration of p53 protein in the cytoplasm by immunohistochemical (IHC) techniques using p53-specific antibodies.
- 2) Mutational analysis of p53, employing standard molecular biological techniques using DNA extracted from microdissected tumor tissues.
- 3) Inactivation of BRCA1 gene due to hypermethylation of BRCA1 promoter in chemically modified DNA, and then using methylation-specific PCR

Thus, in this project we tested the whether breast cancers from AA women, showed a higher frequency of p53 defects in individual tumors than is observed in breast tumors from White women, and whether the changes in p53 are associated with hypermethylation of BRCA1.

## Methods

Tissue blocks were obtained from 94 women who have been diagnosed with breast cancer at Crozer-Chester Medical Center, located near Philadelphia, PA, and the Medical College of Pennsylvania Hahnemann University. In addition, we had obtained tumor samples from New-York Presbyterian Hospital in New York City.

**Immunohistochemical Analysis:** Formalin-fixed, paraffin-embedded archival surgical tissue blocks were identified as corresponding to the appropriate patient charts. The Pathology Departments at MCP and Crozer identified the tissue blocks by slide review, and this along with the pathology chart was forwarded to us. Deparaffinized and rehydrated sections were retrieved in a citrate-buffered solution via heat-induced epitope retrieval for 6 min by microwave. Endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide for 15 min. Nonspecific protein binding was blocked using serum-free protein block (DAKO) for 7 min. A standard avidin-biotin complex procedure was used (ABC, Vector Labs). For identification of tumors showing over-expression of p53 protein, the mouse monoclonal antibody PAb-1801 (Lab Vision) was employed and graded whereby we modified the method of Elledge et al., (1994) using a scale of 0-6, in which positivity is for p53 was consider if score was 3 or more. In this scale, intensity was graded from 0-3 and the proportion of positive cells was 0 < 10%, 1 = 10-32%, 2 = 33-66%, 3 > 66%.

**DNA Extraction from Formalin Fixed Tissue:** Genomic DNA was extracted from paraffin fixed breast tissue sections following a paraffin removal procedure using xylene-ethanol in standardized procedures (Shambrook and Russell, 2001). Aliquots of the purified supernatant were taken directly out of the sample extraction tube and used for PEP reactions; the remaining sample was stored at -20°C.

**PCR-SSCP analysis and direct DNA sequencing for p53 mutations:** The genomic DNA (50-150 ng) was amplified by PCR using oligonucleotide primers designed for TP53 gene from published sequences (Orita et al., 1989; Jhanwar-Uniyal and Gulati, 1998). PCR-SSCP analysis of Exons 4, 5,6,7,8 and 9 of the p53 gene, with nucleotide length of 139 to 330 bp, was performed using a published technique (51). These regions contained domains of p53 highly conserved among species, and they are also the site of frequent mutations in breast cancer. Briefly, 50 ng of genomic DNA was amplified with 0.4 umol/L of forward and reverse primers, dNTPs (2.5 umol/L), 10 mM Tris (pH 8.8)-MgCl<sub>2</sub> buffer and 0.2 units of *Taq* polymerase (Perkin Elmer-Cetus, USA) in a final volume of 25 :1 ul. Conditions for PCR were as described in the literature (Orita et al., 1989; Jhanwar-Uniyal and Gulati, 1998) and amplification was carried out in an automated DNA

Thermal Cycler (Eppendorf). An aliquot of each was diluted with 0.1 % sodium dodecyl sulfate (SDS) and 10 mmol/L EDTA, and further diluted 1:1 with sequencing stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Samples were heated at 95°C for 5 min, chilled on ice, and immediately loaded onto a 6% polyacrylamide (acrylamide: N, N'-bisacrylamide =49:1) in Tris/EDTA/borate buffer, stained with ethidium bromide, and photographed.

### **Methylation of BRCA1 gene:**

#### **Modification of genomic DNA:**

Genomic DNA was modified with bisulfite to convert the cytosine nucleotide to uracil (Esteller et al., 2000). In the reaction, all cytosines are converted to uracil, but those that are methylated (5-methylcytosine) are resistant to this modification. This altered DNA can then be amplified and sequenced to provide detailed information about the region of the methylation of CpG sites for specific genes. DNA (1 ug) was denatured with NaOH for 10 min at 37°C followed by bisulfite treatment and incubation at 50°C for 16 hrs after which DNA was purified, treated with NaOH, and then precipitated with ethanol.

#### **BRCA1 promoter methylation:**

We have used a promoter specific to the BRCA1 gene and specifically designed for the methylated and unmethylated sequence. Modified DNA was amplified with BRCA1 promoter specific primers. PCR conditions were as follows: 96°C for 5 min. then 35 cycles of 96°C for 20 sec., 60°C for 20 sec, 72°C for 90 sec; and finally 5 min at 72°C. Controls without DNA were run for each set of PCRs. The amplified PCR product was electrophorised, stained with ethidium bromide and directly visualized under UV illumination.

## **Results**

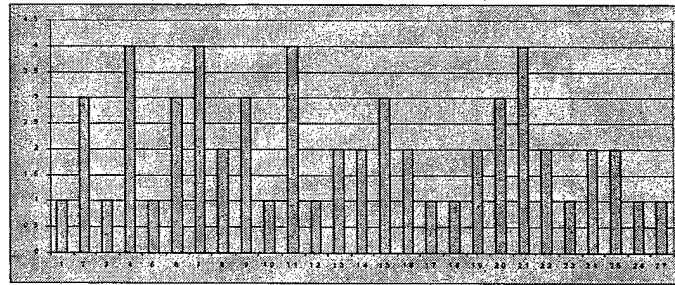
### **Study population:**

Breast carcinoma samples from 34 AA women and 60 WA women from the two hospitals in Pennsylvania, namely Crozer and MCP. Figure1 represents the number of breast cancer cases at age of diagnosis in AW and AA cohorts. The median age of AA was 62 years (range 30-91) and that for AW was 65 years (range:36-75).

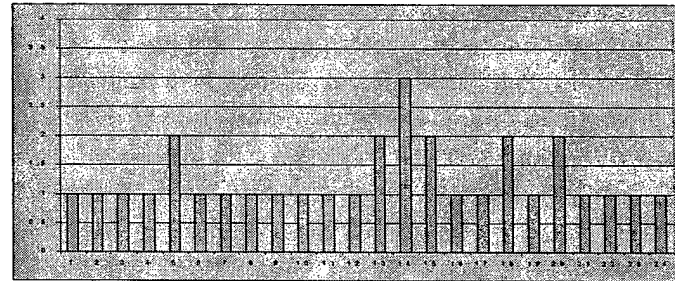
	Race (n)		Age at Diagnosis (years)	
	African American (AA)	American White (AW)	African American (AA)	American White (AW)
MCP	30	11	30-91	43-91
Crozer	4	49	51-74	36-75
<b>Total</b>	<b>34</b>	<b>60</b>	<b>30-91</b> (Median age 62)	<b>36-91</b> (Median age 65)

*Prevalence of Breast Cancer*

American  
White



Africa  
American



**Figure.1: Age at diagnosis (X axis) versus number of patients (Y axis).** As shown in Figure 1, the earliest age at diagnosis was 30 among AA and 36 among the AW. There were 7 breast cancer patients under age 55 years among the 34 for AA (20%) and 22 among the 60 AW (35%).

**Table 1: Distribution of p53 Scores:**

Over-expression of p53 protein was determined by employing immunohistochemistry using the mouse monoclonal antibody PAb-1801, that detects both wild-type and mutant protein. Since normal p53 has a short half-life and is generally undetectable, the expressed levels of p53 are considered to be mutant p53. The levels of p53 are graded by using the method of Elledge et al., (1994) with slight modification. In this, using a scale of 0-6, in which positivity score was 3 or higher. In this scale, intensity was graded as 0-3 and the proportion of positive cells was 0 < 10%, 1 = 10-32%, 2 = 33-66%, 3 > 66%. At the completion of grading both numbers, the proportion of cells expressing p53 and the intensity of expression are combined.

P53 Grading	0	1	2	3	4	5	6
African American (AA)*	7	10	5	2	3	6	1
White American (AW)	22	16	5	6	3	1	4

\* Only n=57 samples were analyzed for AW



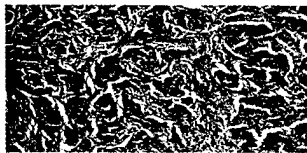
**Table 2: Expression of mutant p53 in African-American and American-White Women: positive and negative scores of P53:**

p53 Negative (Score 0-3)		p53 Positive (Score 4-6)	
AA	AW*	AA	AW*
24	49	10	8
(70%)	(86%)	(29%)	(14%)

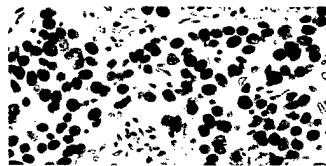
\*Only n=57 samples were analyzed for AW

Accumulation of mutant p53 protein resulted in a higher immunohistochemical signal to the cells in the malignant breast epithelium. These data are presented in Tables 1 and 2. P53 positivity (overexpression and greater intensity), which confers a poor prognosis, is notably more prevalent in AW as compared to AA. Of all samples analyzed, 29% of those originating from AAs displayed p53-positive staining, while only 14% of those from AW displayed the high score of p53. Moreover, 86% of AW showed low p53 staining while 70% of AA showed low staining. It is interesting to note that a 0 score was seen in 20% of all AA, while 39% of AW breast samples displayed zero scores. A strong p53-positive staining indicates the presence of mutant p53, while absence of p53 means that wild-type p53 is present.

**Figure 2:**



H&E staining in paraffin-embedded human breast carcinoma



Immunohistochemical staining of p53 in paraffin-embedded human breast carcinoma using p53 antibody 1801, showing intense nuclear staining of p53

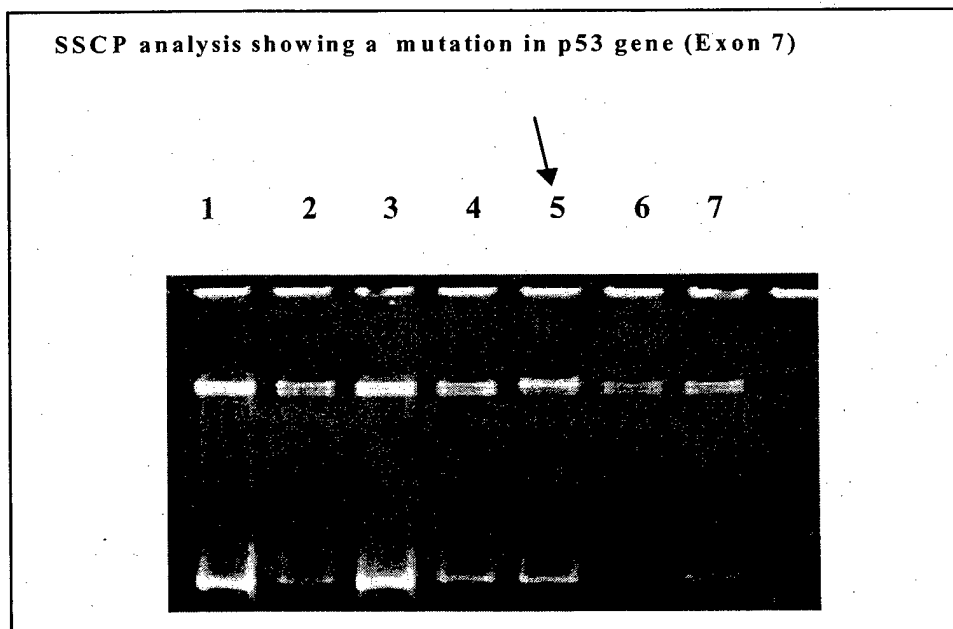


Immunohistochemical staining of p53 in paraffin-embedded human breast carcinoma using p53 antibody 1801, showing absence of p53 staining.

**p53 Gene mutation analysis:** Mutational analysis of the p53 gene was carried out in samples that displayed high scores of p53 immunostaining (Scores 4-6). Some samples that showed p53-negative staining but were ER and PR negative were also studied for p53 mutation. PCR-SSCP analysis of these samples demonstrated a strong link between high scores in p53

immunohistochemical analysis and p53 mutation. So far we have done p53 gene analyses only on Exon 5-9, also known as the hot-spot zone of the gene. Samples from 2 AA subjects, that displayed a high score of p53 immunohistochemistry, had mutations of p53 in Exon 7 (Figure 3). Both these subjects had infiltrating invasive ductal carcinoma. One of these two subjects was diagnosed with breast cancer at the relatively young age of 30 and had negative ER and PR status. One other sample from an AA subject with medullary carcinoma displayed high p53 IHC and showed a mutation of the p53 gene in Exon 5. Thirty percent of the samples displaying high IHC for p53 protein, from AA origin, showed mutation in the p53 gene (Exon 5-9 studied so far). We detected no mutation in Exon 5-9 in six samples that displayed high p53 IHC. However, we detected two p53 mutations in an AW-derived sample. In addition, we observed a p53 mutation in Exon 5 from DNA derived from an AW, this case had shown a border line ER and PR, and had a high proliferative index.

**Figure 3:**



Determining the mutations in Exons 2-4 and 10-11 of p53 in breast cancer samples that display high scores for p53 levels using IHC but failed to demonstrate mutation in the hot-spots zone (Exon 5-9), will be important in establishing the role of p53 in breast cancer in AA women.

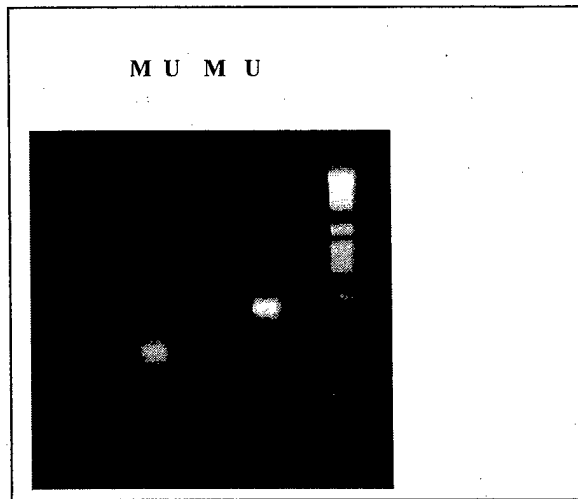
#### **Methylation of BRCA1 Promoter:**

Our results to date show hypermethylation of BRCA1 promoter (Figure 4) in samples that display p53 mutation. Three samples, from AA women had high p53 expression and two of these three had p53 mutation in Exon 7 (one is still being studied for Exons 2-4, and 10,11). Notably, two of these subjects had onset of breast cancer at a relatively very young age (30 and 33 years). We had also observed hypermethylation of BRCA1 in a sample from AW women; two of them showed ER and PR negative status. An example of a BRCA1-hypermethylated sample is shown

in Figure 4. As shown in this figure, bisulfite-modified DNA was amplified with two sets of primers (methylation-specific and unmethylation-specific), first sample (from left) shows presence of methylated (M) band and absence of unmethylated (U) and second sample showed no methylated band but shows a unmethylated allele. This epigenetic mechanism of gene inactivation of the BRCA1 gene is observed in sporadic cancer and may explain an inactivation of a tumor suppressor gene besides mutation or loss of heterozygosity (LOH).

M=Methylated; U=Unmethylated

**Figure 4**



#### **Discussion:**

We have confirmed histopathological diagnosis in H&E-stained sections from the tumor blocks. Immunohistochemical determination of p53 is completed and graded. Expression of abnormal p53 in this study was greater in AA than in WA women. An inverse correlation exists between the premenopausal age and levels of abnormal p53 ( $r = -0.73$ ) only in AA women, which indicates that the lower the age the higher the p53 levels. Mutational analysis of Exon 5-9 of p53 gene shows that number of p53 mutation were higher in AA than AW women.. Mutational analysis of other Exons (2-4 and 10 and 11) of the p53 gene is currently underway. Hypermethylation of the promoter region of the BRCA1 gene was seen predominantly in cases with mutated p53 and negative status of ER and progesterone receptors. To date, our data suggest that a racial disparity exists in-terms of genetic factors which may contribute to poorer prognosis of breast cancer in AA women.

#### **KEY RESEARCH ACCOMPLISHMENTS**

- Median age at onset of breast cancer was 62 years in AW and 65 in AA.
- Although onset of breast cancer was at a much younger age in AA, the incidence of breast cancer in AW was generally greater at age below 55.
- Expression of abnormal p53 in this study was greater in AA than in WA women.

- Expression of abnormal p53 was always higher in samples with loss of estrogen and progesterone receptor positivity.
- The number of p53 mutations was greater in the AA than in the AW women.
- An inverse correlation exists between the premenopausal age and levels of abnormal p53 ( $r = -0.73; p < 0.05$ ) only in AA women, which indicates that the lower the age the higher the abnormal p53 levels. No such correlation was seen in AW women ( $r = -0.09$ ; NS).
- A strong association was seen between presence of abnormal p53 and hypermethylation of the BRCA1 promoter.

## REPORTABLE OUTCOMES

The outcome of this study strongly suggests that p53 abnormality in AA women, beginning during their premenopausal years, may contribute to a more virulent form of breast cancer in AA women. An inverse relationship between age and abnormal p53 in AA women indicates that AA women have an increased frequency of defects in p53 gene and these defects may contribute to the poor outcome of disease. However, inactivation of BRCA1 in both AA and AW was associated with a biological phenomenon where a common mechanism for silencing gene expression occurs via hypermethylation of the promoter region-associated CpG island. A similar mechanism is involved in the loss of estrogen receptor (ER) positivity, which is particularly common in premenopausal AA women, and has also been associated with BRCA1 inactivation and p53 overexpression or p53 gene mutation. In fact, we found that a subset of breast cancer patients with specific histological type, such as, Medullary carcinoma, from both AA and AW patients, had ER negative status with overexpression of p53 and hypermethylation of the promoter region of BRCA1 gene.

## CONCLUSIONS

The outcome of this study is very important, and the results are very impressive, although they require further study to fully explain the variations in genetics that relate to breast cancer in AA women. Furthermore, the finding of this study will help design unique diagnostic and treatment strategies in this specific group. The identification of altered BRCA1, with p53 abnormalities, that are present in excess in breast cancer of AA women provides a rationale for a future prospective clinical study. This will be designed with sufficient statistical power to determine whether the loss of BRCA1 protein, with or without altered p53, is an independent prognostic biomarker. The outcome of this study is to provide a novel means of developing targeted therapeutic agents.

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**Appendices:**


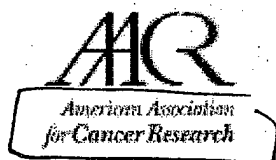
- 1) Based on our findings, we have submitted an abstract to Annual Meeting of American Association for Cancer Research (AACR;2003).
- 2) Submitted Abstract (2004)
- 3) Two Articles where this grant is acknowledged.
- 4) Manuscript in preparation.

**#4937 Involvement of p53 and BRCA1 genes in breast cancer in African-American and white women.** Meena Jhanwar-Uniyal, Gina Day Stephenson, Renee Royak-Schaler, Chung-Xiou Wang, Mohanrao Achary, Anthony P. Albino, and John Whysner. *Institute for Cancer Prevention (American Health Foundation-Cancer Center), Valhalla, NY and Albert Einstein College Of Medicine and Montefiore Medical Center, Bronx, NY.*

The p53 and BRCA1 tumor suppressor genes play a key role in establishing genome stability. Inactivation or mutation of p53 is seen in variety of cancers

including breast cancers. There have been discrete p53 mutations in tumors African-Americans (AA) that were different from those observed in White Americans (WA). Germline mutations in BRCA1 have been reported in hereditary cancer, but somatic mutations of BRCA1 gene are absent in sporadic cancer. Recent studies have shown that inactivation of BRCA1 occurs via the hypermethylation of the promoter region of the BRCA1 gene. The purpose of this study was to clarify the role of two susceptibility genes as determinants of potential differences of outcome differences in African-American and White women diagnosed breast cancer. AA breast cancer patients more frequently have clinical and pathological features of advanced disease and reduced survival than their white counterparts. Well-characterized tumor tissues from 94 breast cancer patients (34 AA, 60 WA) diagnosed at two Philadelphia hospitals were screened for mutations of p53 and BRCA1 inactivation using various methods including immunohistochemistry, DNA-modification followed by methylation-specific PCR, PCR-SSCP, and a direct DNA sequencing. Our results show that: 1) the number of p53 positive cases were lower in AAs as compared to WAs, 2) p53 overexpression of its protein was more commonly observed in AAs than WAs, and also the number of p53 mutations were greater in AAs compared to WAs, 3) hypermethylation of the promoter of BRCA1 gene occurred in cases where p53 was mutated with concurrent negative estrogen receptor (ER)/progesterone receptor (PR) status in both AA and WA tumors. No BRCA1 inactivation was detected among the patients without p53 mutations or in those with p53 mutations and ER/PR positive tumors. We hypothesize that loss of expression of ER and PR proteins leads to genomic instability that may result from the inactivation of p53 via mutation and BRCA1 hypermethylation. Data from this study suggest that variation in these two susceptibility factors may have prognostic significance in AA and WA breast cancer patients. (Supported by NCI CA 17613, DAMD-17-99-1-9055 (M.A.), DAMD-17-00-1-0675 (M.J.U.))



 [Print this Page for Your Records](#)[Close Window](#)**Control/Tracking Number :** 04-AB-5779-AACR**Activity :** Abstract Submission**Current Date/Time :** 11/7/2003 7:46:28 PM**Metastasis to Brain: Gene Expression Profiling**

*David H. Harter, Anthony P. Gulati, Deborah L. Benzil, Gregory Khitrov, Myron R. Melamed, Raj Murali, Meena Jhanwar-Uniyal.* New York Medical College, Valhalla, NY, Institute For Cancer Prevention, Valhalla, NY, The Rockefeller University, New York, NY

The main model of metastasis maintains that most primary tumors have low metastatic potential, however rare cells within the primary tumors acquire metastatic capacity through somatic mutation (Fidler, Nature, Reviews Cancer, 3; 2003). The most common brain metastatic phenotype has the ability to migrate from the primary tumor, survive in blood, pass through the blood brain barrier, invade distant tissue, and form blood vessels to establish itself as a metastatic tumor. Most of the studies of metastasis have focused on gene expression profiling of metastatic tumors to organs such as lymph node, liver and bone. Characteristic genetic changes underlying the metastatic progression of malignant adenocarcinoma to the brain, however, is not fully understood. The goal of this study was to explore specific gene alterations occurring in brain metastases. Microarray analysis was performed in tissues of metastatic tumors excised from the brain using a 22k-gene chip. The primary site of the tumors was the lung. Data analysis was performed using IOBION Genetraffic software, with 2-fold up or down gene expression cutoff. Selected microarray results were verified by RT-PCR and Western-blot analysis. Among the 22,000 genes examined, 125 genes were consistently upregulated or downregulated in these metastatic tumors. Upregulated genes were considerably more numerous than those that were down-regulated. Examination of cell adhesion- and migration-related genes revealed up-regulation of actin, biglycan, myosin light chain kinase, integrin beta2 and 6, integrin alpha V, fibronectin, Rho GTPase-activating protein and Laminin beta 4. Increased expression of TGF- $\beta$  and its related genes was evident. Up-regulation of oncogenes, such as the platelet-derived growth factor beta Met proto-oncogene (hepatocyte growth factor) and RAB2, was also seen. The angiogenic mediator vascular endothelial growth factor (VEGF) expression was upregulated in the metastatic tumor. Most of the DNA repair genes were downregulated, however, three prime repair Exonuclease 2 (TREX2), which is involved in DNA replication, repair and recombination, was markedly upregulated. Among the down-regulated genes, the neuroprotective heat shock protein 70 (Hsp70) was noticeably down-regulated in metastatic tumors. The results of this study demonstrate that those genes that are involved in adhesion, motility and angiogenesis are upregulated in metastatic tumors, thus making metastasis-prone cells more susceptible to migration, homing, angiogenesis and tumor growth. (Supported by NCI CA 17613 and DAMD17-00-1-0675).

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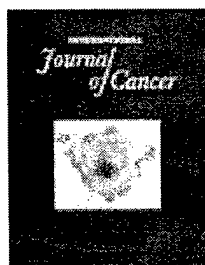
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**International Journal of Cancer****Early View** (Articles online in advance of print)

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Hui Zhao <sup>1</sup>, Meena Jhanwar-Uniyal <sup>2</sup>, Prasun K. Datta <sup>3</sup>, Srishailam Yemul <sup>4</sup>, Lap Ho <sup>4</sup>, Gregory Khitrov <sup>5</sup>, Ilya Kupersmidt <sup>6</sup>, Giulio M. Pasinetti <sup>4</sup>, Tarun Ray <sup>7</sup>, Raghbir S. Athwal <sup>8</sup>, Mohanrao P. Achary <sup>1\*</sup>†

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**Abstract**

Metastases of various malignancies have been shown to be inversely related to the abundance of nm23 protein expression. However, the downstream pathways involved in nm23-mediated suppression of metastasis have not been elucidated. In the present

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investigation, we used cDNA microarrays to identify novel genes and functional pathways in nm23-mediated spontaneous breast metastasis. Microarray experiments were performed in a pair of cell lines, namely, C-100 (only vector transfected; highly metastatic) and H1-177 (nm23 transfected; low metastatic), derived from human mammary carcinoma cell line MDA-MB-435. The cDNA microarray analysis using GeneSpring software revealed significant as well as consistent alterations in the expression (up- and downregulation) of 2,158 genes in a total of 18,889 genes between high and low metastatic cells. Some of these genes were grouped into 6 functional categories, namely, invasion and metastasis, apoptosis and senescence, signal transduction molecules and transcription factors, cell cycle and repair, adhesion, and angiogenesis to extrapolate an association between these genes and different functional pathways involved in nm23-regulated metastasis. The results suggest that nm23 gene plays a major role in metastasis and its mechanism of action of metastasis suppression may involve downregulation of genes associated with cell adhesion, motility (integrins  $\alpha 2$ , -8, -9, -L and -V, collagen type VIII  $\alpha 1$ , fibronectin 1, catenin, *TGF- $\beta$ 2*, *FGF7*, *MMP14* and 16, *ErbB2*) and possibly certain tumor/metastasis suppressors (2 members of *SWI/SNF*-related matrix-associated proteins 2 and 5 and *PTEN*). © 2003 Wiley-Liss, Inc.

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## BRCA1 IN CANCER, CELL CYCLE AND GENOMIC STABILITY

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### 1. ABSTRACT

The BRCA1 gene was isolated in 1994; germline mutations of this gene are known to confer susceptibility to breast and ovarian cancer in high-risk families. Since its discovery, several mutations have been identified in this gene; these are scattered throughout the gene, and include insertion and deletion frameshifts, base substitutions, and inferred regulatory mutations. Its role in the pathogenesis of breast cancer, which accounts for almost 95%, although unproven to date, cannot be ruled out. The functional inactivation of both copies of this gene in sporadic tumor cells does not follow the traditional mode: the loss of function in BRCA1 is not accompanied by underlying mutation of the gene in tumor cells with loss of heterozygosity for the BRCA1 gene. Several studies now suggest that an alternate mechanism of inactivation, involving promoter hypermethylation that results in reduced expression of the gene, may be common to a significant proportion of sporadic breast and ovarian cancers. BRCA1 as a tumor suppressor plays an important role in maintaining genomic stability. BRCA1 has the ability to interact with numerous proteins and to form complexes that are involved in recognizing and subsequently repairing DNA. BRCA1 contains several functional domains that directly or indirectly interact with a variety of proteins via protein-protein interaction; these include tumor suppressors (BRCA2, p53, Rb and ATM), oncogenes (c-Myc, casein kinase II and E2F), DNA damage repair proteins (RAD50 and RAD51), cell cycle regulators (cyclins and cyclin dependent kinases), transcriptional activators and repressors (RNA polymerase II, RHA, histone deacetylase complex and CtIP), DNA damage-sensing complex and mismatch repair proteins (BRCA1-Associated Surveillance Complex; BASC) and signal transducer and activator of transcription (STAT) among others. Formation of foci containing BRCA1 by inherited mutations, or epigenetic mechanisms (promoter methylation) in sporadic cancers leads to a loss of DNA repair ability, disrupts the potential to form complexes with other proteins that are crucial for DNA repair pathways. Thus, BRCA1 plays a significant role in maintaining

genomic stability and serves as a tumor suppressor in breast cancer tumorigenesis.

### 2. INTRODUCTION

#### 2.1. Mutation spectrum of BRCA1-associated tumors

The American Cancer Society estimated that a total of 203,500 new cases of breast cancer (out of 647,400 estimated cancers at all sites) would occur among US women in the year 2002 and assesses the probability that one in eight American women would develop breast cancer during their lifespan (American Cancer Society Facts and Figures, 2002). Taken together, cancers of the breast and ovaries constitute almost one fourth of all cancer-related mortality in this country. BRCA-1 and BRCA-2 genes (1,2,3) are known to be associated with early onset familial breast and ovarian cancer. Patients with a strong hereditary component account for only 5% of all breast cancers occurring in the United States (4,5); nevertheless, identification of genes responsible for hereditary cancers is important, as such genes have been shown to play a critical role in the much more common form of "sporadic" tumors (6) in a variety of cancers. The role of the BRCA1 gene in sporadic breast cancer, however is not well defined, as mutations of these two genes in tumors with loss of heterozygosity (LOH) for BRCA1 and 2 are very rare (6). As reviewed by Szabo and King (1995) (4), BRCA1 and BRCA2 combined contribute to only 6-10% of breast and ovarian cancer regardless of the family history. In addition, approximately 30% of high-risk families do not exhibit mutations in either BRCA1 and BRCA2 genes. Such observations are consistent with the fact that there may be other genes that may predispose individuals to breast cancer. A limited number of recurring mutations (BRCA1 185delAG, 5382insC; BRCA2 617delT) in the BRCA 1 and 2 genes account for a substantial fraction of the breast cancer burden in the Jewish population (4,7).

Molecular studies on hereditary cancer syndromes, such as retinoblastoma, adenomatous polyposis

coli, etc., have demonstrated that at least two genetic events are necessary for the development of tumors. One of these events must be mutation in the tissue-specific cancer-predisposing gene, whereas second event results in inactivation of the corresponding normal allele, which is normally accomplished by LOH.

A wealth of information is now available regarding the extent and type of germline mutations detected in various populations in the world. These studies clearly demonstrated that mutation in the BRCA1 gene are not localized to a specific exon.

The first breast cancer susceptibility gene discovered BRCA1, spans approximately 100 kb on the long arm of chromosome 17 (17q21.3) from which a 7.8-kb mRNA is transcribed that encodes a protein of 1863 amino acids (8), and consists of 24 exons, including a very large exon 11(8). The tumor suppressor gene BRCA1 was cloned through its linkage to inherited breast cancer (8).

BRCA1 is well conserved among species. Approximately 50% of inherited breast cancer cases are the result of germ-line mutation in the BRCA1 gene, and virtually all families have a history of both ovarian and breast cancer carry mutation in the gene (6,9)

Hereditary breast cancers account for 10-15% of all breast cancer cases whereby ~50% of these are associated with the susceptibility genes BRCA1 and BRCA2. Mutations in the BRCA1 gene are rare in sporadic breast cancer. LOH and mutation in BRCA1 has been observed in ovarian cancers (10). Loss of the wild-type allele is often seen in the tumor of a carrier with germline BRCA1 mutation, qualifying BRCA1 as a tumor suppressor gene. Inheritance of a mutated copy of the BRCA1/2 genes increases the lifetime risk of breast cancer 5-to 8-fold and that of ovarian cancer 20-to 40- fold. Genetically-predisposed individuals typically present with cancer at an earlier age, i.e., >50% of BRCA1 mutant carriers develop cancer by age 50. Members of breast cancer-prone families often seek genetic counseling to assess their relative risk for cancer development. High-risk patients are then evaluated by identifying germline mutations in the BRCA1 (and/or the BRCA2) gene. Most of these individuals carry a nucleotide sequence alteration in the BRCA1 gene (~80%) that results in a frameshift or missense mutation, whereas a subset of patients (~10%) possess chromosomal rearrangements affecting the gene. A third subset, ~10% of high-risk patients, lack discernable mutations in either BRCA1 or BRCA2, despite a calculated high probability for mutation on the basis of family history. Recent studies show that methylation-dependent epigenetic silencing of BRCA1 can contribute to the development of breast cancer that is indistinguishable from that of patients with BRCA1 mutation.

### 2.2. Mutation spectrum and phenotypes of BRCA1-associated tumors

BRCA1-associated tumors are largely ductal type, histological grade III, and show significant lymphocytic infiltration (11). BRCA1-associated tumors

display distinct histological and biological features indicating that these tumors are under distinct genetic control. A strong correlation is seen between the position of a BRCA1 mutation and the ratio of breast to ovarian cancer incidence within the families (12). In particular, mutations located upstream of exon 13 are more likely to give a high proportion of ovarian and breast cancer while mutations at the 3' end of this exon give an opposite phenotype. Furthermore, BRCA1 mutations at the 5' or 3'-ends of the coding region correlate with highly proliferative tumors (13). This may suggest that inactivation of a functional domain of the BRCA1 protein, such as the RING finger or BRCT domain, results in a more severe phenotype than mutation occurring somewhere else. Since the discovery of the BRCA1 gene in 1994 (8), about 113,705 cases have been reported in OMIM (Online Mendelian Inheritance in Man). Unique coding region mutations have been identified as listed in the Human Gene Mutation data base ([www.nhgri.nih.gov/intramural.research](http://www.nhgri.nih.gov/intramural.research)). Almost all kinds of mutations seen in the BRCA1 gene, include inversion, deletion, insertion, missense/nonsense and aberrant splicing. Most of these caused frameshift of the coding region leading to a truncated BRCA1 protein. The relatively low number of point mutations found in BRCA1 suggest that a significant portion of the encoded protein has to be inactivated to confer susceptibility to breast cancer. Most of the tumor-associated point mutations are found in conserved domains such as the RING finger and the BRCT domain. No obvious signs of clustering or mutational hotspots have been seen in BRCA1, but certain sequences and nucleotides of BRCA1 may be more susceptible to mutations than others (14). Certain polymorphisms and unclassified variants also have been reported. Most mutations generate a premature stop codon, which results in the production of a truncated protein. Many BRCA1 mutations occur in repeated motifs, including strings of homonucleotides, short direct repeats, and inverted repeats. A good example is the mutation 185delAG, prevalent among Ashkenazi Jews, which precedes a second AG pair (TCTTAGAGTGTGTC) (15). The allelic frequency of this mutation (185delAG) in the Ashkenazi Jew population in USA is about 1% (16). However, among Ashkenazi Jews in Australia the prevalence of this breast carcinoma-predisposing allele is even greater (17). This mutation as well as BRCA1 5382incC are considered to be founder mutations, associated with the migration of carriers with this mutation. The length of the Ashkenazi Jewish founder mutation, 185delAG, has been estimated to be 760 years old (18). Similarly, among African-Americans, three recurrent BRCA1 mutations, namely 943ins10, 1832del5, and 5296del4, have been described and it has been considered that these are likely to arise from a common ancestry (reviewed by Olopade *et al.*, 2003) (19). Similarly, many recurrent mutations have been described in many populations. These mutations may occur due to slippage and misalignment in DNA replication. The physiological and biological alterations caused by this mutation can lead to breast cancer. The individual heterozygote for germ-line mutation in BRCA15382incC developed breast cancer at a relatively early age. A breast tumor cell line (HCC1937; ATCC) that is homozygous for BRCA1 5382insC mutation (20), has been used to

decipher the physiological role of BRCA1 in DNA repair pathways. These aspects are discussed later in this chapter. There have been cases of breast cancer-prone families where rearrangements involving 3.4 and 11.5 Kb of the BRCA1 gene resulted in loss of amino acid in the C-terminus of the gene, which is involved in DNA repair pathways (21,22). Since then, many more novel ethnic mutations have been described.

Recent studies have suggested that inherited BRCA1 mutations are able to promote an oncogenic event, perhaps by masking the functions of the remaining wild-type BRCA1 allele (23). Mutations in BRCA1 gene sporadic cases are absent or rare. Nonetheless, BRCA1 has been shown to play a role in the development of breast cancer in sporadic breast cancers. In case of early onset of breast cancer without a family history, germline mutation of the BRCA1 gene has also been reported (6). Sporadic breast cancers account for about 90% of all cases, with hereditary breast cancer accounting for the balance. A great many studies have examined the molecular pathogenesis of sporadic and hereditary breast cancer, but very few have examined the epigenetic contributions to this process. The possible contribution of methylation-dependent epigenetic regulation of BRCA1 in sporadic and hereditary breast cancer remains to be determined. BRCA1-associated tumors are generally estrogen Receptor (ER-) negative (24,25). Some studies have shown that loss of ER positivity in tumors is due to methylation of ER, specifically of ER- $\alpha$ . ER- $\alpha$ -negative cancers arising in BRCA1 mutation carriers were more extensively methylated than ER- $\alpha$  negative cancers from women without a BRCA1 mutation (26).

### 2.3. Methylation in the promoter region of BRCA1

In many sporadic breast cancers methylation of the BRCA1 promoter has been reported. In non-hereditary breast cancers, which account for 90% of cases, the involvement of BRCA1 has not been so clearly elucidated. A great variety of genetic mutations was found in BRCA1-linked families. Inactivation of a tumor suppressor gene by loss of one allele, linked to mutation of the remaining allele, is a common mechanism. BRCA1, in some ways, puts itself on the brink of ignoring the two hit model proposed by Knudson that is generally followed by other tumor suppressor genes; because no sporadic mutations was seen in BRCA1, an alternative mechanism to genetic mutations, namely hypermethylation of the BRCA1 promoter resulting in a loss of expression, has been shown. Methylation of DNA represents a significant epigenetic alteration in humans. It occurs most frequently on the 5'-methylcytosine residue of 5'-CpG-3' dinucleotides. De novo enzymatic methylation of 5'-CpG islands can lead to the inactivation of the gene. CpG sites are generally clustered into islands, called "CpG islands", usually cover 0.2 to several KB and are found in the promoter region of the genes (27). Enzymatic methylation of 5'-CpG islands can lead to the inactivation of the contiguous gene. DNA methylation inhibits transcription by interfering with transcription initiation. This repression can arise by several means.

In human cancer, this epigenetic (non-genetic) event has been shown to be a powerful mechanism by which

tumor suppressor gene activity is inhibited. Local promoter hypermethylation in human cancer is often part of global genomic hypermethylation. DNA methylation inhibits transcription by interfering with transcription initiation. Thus the potential mechanism is a reduction of binding affinity of sequence-specific transcription factors. The BRCA1 gene is regulated by two promoters. Two distinct transcripts differing by the alternative use of the first exon have been described (28). BRCA1 promoter lacks TATA, but has several CAAT boxes, GC boxes and PEA3 binding sites, and a CREB binding site (29,30). Sequence homology searches reveal that a classic estrogen response element (ERE) sequence was not present in either BRCA1- $\alpha$  or BRCA1- $\beta$  promoter regions (31). An alternative ERE was observed in BRCA1  $\beta$  promoter (30) which is believed to be responsive to estrogen stimulation via the classical ER pathway to stimulate transcription. Estrogen may regulate BRCA1  $\alpha$  promoter via some complex or indirect mechanism(s) since BRCA1- $\alpha$  lacks a conventional ERE. A putative AP1 site is present in the BRCA1 promoter (28,32). Several CpG sites exist in BRCA1 promoter; the 5' CpG island of BRCA1 encompasses about 2 Kb. The region of the BRCA1 promoter shares the first exon of another gene, NBR2, and its bi-directional promoter with exon 1a and 1b of BRCA1 (28,33). Hypermethylation of BRCA1 promoter has been detected in about 11-31% of breast cancer cases and about 5-15% of ovarian cancer. Several methods have been used to identify methylation, namely Southern analysis with methylation-specific PCR (MSP) and sodium bisulfite, followed by PCR. Normal tissues or cell lines did not show BRCA1 methylation; it has been observed exclusively in malignant breast and ovarian tissues. BRCA1 hypermethylation varies according to histological subtypes and is common in mucinous and medullary subtypes (34). Interestingly, these histological sub-types are also highly represented in inherited BRCA1 mutant (35). These cancers display a distinct phenotype, such as loss of ER and PR positivity, and often have mutated p53 (25). Hypermethylation was more frequent in high-grade breast cancer (24,36). There is a strong correlation between promoter hypermethylation and decrease in gene expression and protein expression (37). On the other hand, in one study, 37 tumors that showed a reduction in BRCA1 expression gave no evidence of hypermethylation (38). This indicates, that a mechanism other than methylation is also responsible for suppression of BRCA1 expression. Methylation specifically inhibited the binding of the CREB protein to the CRE site within the 5' regulatory region of the BRCA1 promoter (39). The CRE site appears to play a constitutive role in BRCA1 expression. One possible mechanism by which methylation could abrogate gene expression is by impairing the interaction of transcription factors with DNA binding sites. In this regard the putative CREB binding motif present in  $\alpha$  promoter of BRCA1 has been shown to be sensitive to methylation (37). The 5' SmaI site was found to be in close proximity to a Sp1 binding motif (28) and it has been suggested that the Sp1 element plays a role in protecting CpG islands from de novo methylation (40). A strong correlation has been observed between loss of ER positivity and BRCA1 methylation (41,42). In a study of 96 sporadic breast cancers, 10 out of 11 BRCA1 methylated cases were ER and PR negative (ER- and PR-). On the

other hand, another 31 of 96 cases, that were ER- and PR-failed to show methylation of BRCA1. In our study, we found all ER- and PR- cases to be BRCA1 methylated. It is interesting that the ER CpG island has been found to be methylated in 25% of ER- breast cancers (43), and the same percentage of ER- cases was found to be hypermethylated in the BRCA1 promoter region (41). An abnormal methylation was detected in approximately ten percent of sporadic breast tumors, it indicated that this mechanism alone cannot account for the reduction in BRCA1 mRNA levels observed in the majority of sporadic invasive breast cancer cases (44).

It would be intriguing to see whether a strong correlation exists between loss of ER positivity and BRCA1 hypermethylation. Reduced BRCA1 expression levels in sporadic cancer are strongly correlated with negative ER status (45). Furthermore, an increased DNA methyltransferase activity in ER- cell lines compared to ER+ cell lines, suggests that loss of ER positivity is due to increased methylation. This may mean that methylation of the BRCA1 promoter in ER- tumours may also be secondary to increased DNA methyltransferase activity. BRCA1 expression was low or completely lost in invasive carcinoma, however an opposite, namely higher expression was seen in non-invasive breast cancers (45). The highest levels were observed in samples from non-comedo ductal carcinoma *in situ*, a premalignant breast lesion with a finite, but relatively low rate of progression to invasion. Determination of hypermethylation of BRCA1 may play an important role in early detection of tumors. It has been observed recently that one case of sporadic breast cancer, misclassified for a BRCA1 mutation, turned out to be hypermethylated BRCA1 (46). These investigators used microarray analysis to identify genes associated with BRCA1 hereditary tumors that could contribute to BRCA1 positivity of breast tumors. Direct interaction of BRCA1 with known DNA methyltransferase, or demethylase complex has not been demonstrated. However, BRCA1 has been shown to be physically associated with a component of the histone deacetylase complex (47). It is possible that histone acetylation and CpG methylation may be inter related epigenetic processes. It is also feasible that methylation reflects gene activation rather than being the cause of it. Thus, restoring gene expression by treating cells with the demethylating agent 5' aza-deoxycytosine could restore BRCA1 expression. Such studies remain to be explored.

### 2.4. Physiological role of BRCA1

It is a phospho-nuclear protein, generally residing in the nucleus; however, BRCA1 may "shuttle" between nucleus and cytoplasm (48). BRCA1 consists of the N-terminal Really Interesting New Gene (RING) finger domain and a C-terminal acidic domain termed BRCA1 C terminus (BRCT) (8;49). BRCA1 has both nuclear localization signals (NLSs), nuclear export signals (NES), and 2 C-terminal BRCT domains of about 100 residues (50). BRCT domains are a common protein-protein interaction motif involved in DNA damage response and repair. BRCA1 may participate in mammalian heat shock response pathways (51). BRCA1 has been shown to localize in the mitotic centrosome, where it interacts with  $\gamma$ -

tubulin (Liu *et al.*, 2002), suggesting its role in mitosis. BRCA1 is shown to be associated with a hyperphosphorylated form of retinoblastoma protein (pRb), an interaction that is crucial for BRCA1-induced growth arrest in the G1/S phase of the cell cycle (53). pRb interacts with E2F to inhibit cell proliferation and it is possible that BRCA1 keeps RB in the hyperphosphorylated state to achieve growth arrest. Furthermore, the BRCA1-RB complex interacts with a histone-deacetylase complex (47) to suppress the transcription of E2F-responsive genes, perhaps to inhibit cell growth.

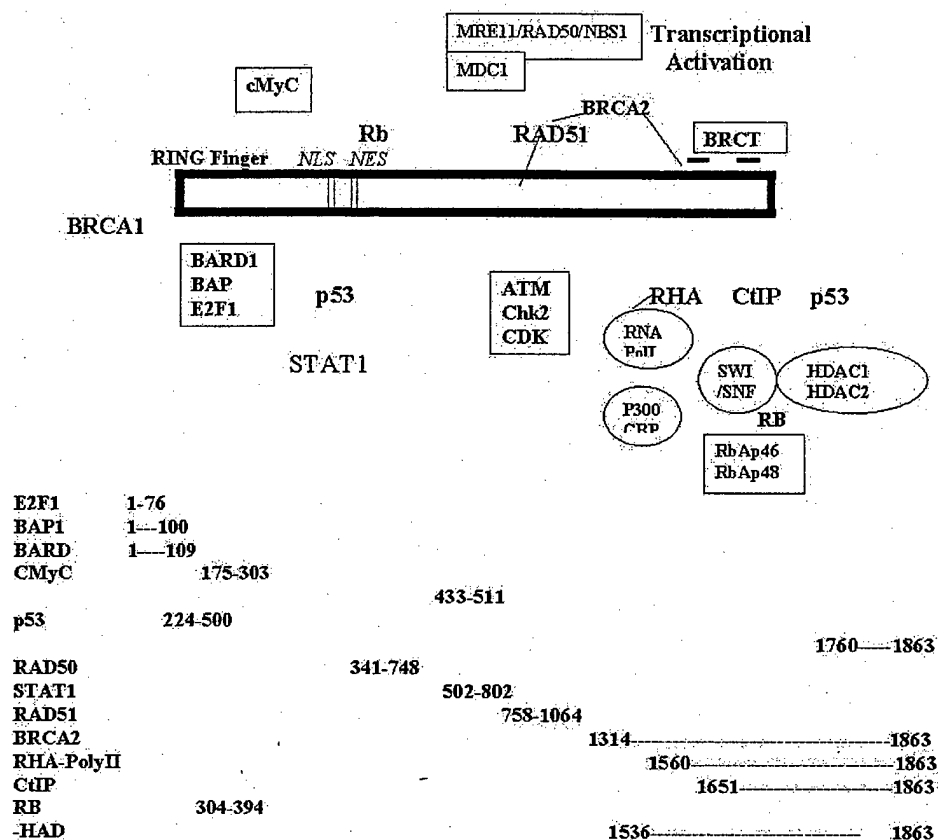
The BRCT domain is found in various proteins, including 53BP1, RAD9, RAD4, crb2 and RAP1(54). The recently described MDC1 (mediator of DNA damage checkpoint protein 1) also possesses a BRCT domain (55). The NH2-terminal RING domain of BRCA1 mediates association with protein BARD1, which is similar in structure to BRCA1 (56) and BAP1 that binds to the wild-type BRCA1-RING finger, but not to germline mutants of the BRCA1-RING finger found in breast cancer kindreds (57; 58). The BRCA1 and BARD1 complex was shown to exhibit ubiquitin ligase activity, which is lost by mutations in the BRCA1 gene in the RING finger region (59,60,61). In fact, recent studies have indicated that BARD1 plays a critical role in preventing nuclear export of BRCA1 by masking NES (48). BRCA1-BARD1 is the first example of a RING-dependent Ubiquitin Ligase that depends on the heterodimer to exhibit ubiquitin activity.

BRCA1 has a protein interaction domain for p53 (62), and BRCA1 expression is modulated by p53 (63). Levels of BRCA1 are down-regulated in response to p53 induction by DNA damage in cells that undergo either growth arrest or apoptosis (63). It has been suggested that, once phosphorylated, BRCA1 acts synergistically with p53 to cause cell cycle arrest after DNA damage; it then is degraded in a p53-dependent manner, when it is no longer required. BRCA1 initially participates in the accumulation of p53 protein but later p53 acts to reduce BRCA1 expression, perhaps via a feedback loop (64,65).

### 2.5. BRCA1 in Cell Cycle Control and DNA Repair

BRCA1, also called the caretaker of the genome (66), is involved in maintaining genome stability by virtue of its important role in cell cycle control, DNA double strand break repair, and transcription-coupled repair (reviewed by Deng and Brodie, 2000(50)). The BRCA1 RING domain has a direct link with Ub ligase, and mutations in these regions of BRCA1 have been shown to predispose to cancer perhaps by altering the Ub ligase activity (61,67). The BRCA1 protein and its interacting proteins are shown in Figure 1.

The BRCA1 protein shows no homology to any known protein and it is expressed widely (8). The functional motifs in the BRCA1 protein that have been described include a RING finger domain, a carboxy-terminal domain called BRCT, binding sites for tumor suppressor p53 and DNA repair protein RAD51, a human homolog of RecA. BRCA1 physically interacts with the proto-oncogene, c-Myc, and it may function as a tumor



**Figure1.** Diagrammatic representation of the BRCA1 protein. The interacting proteins are shown. (See text for detail also see Ref. 50, 77,78).

suppressor by regulating the activity of c-Myc (68). The region of BRCA1 between amino acids 502 and 802 interacts with the C-terminal transcriptional activation domain of the signal and activator of transcription 1(STAT1) and this interaction contributes to its IFN- $\gamma$  activation (69). Each domain interacts with specific protein(s) pivotal for distinct functions in cellular processes. Thus, BRCA1 may directly control various assigned functions and also influence these functions by modulating their interaction with other proteins.

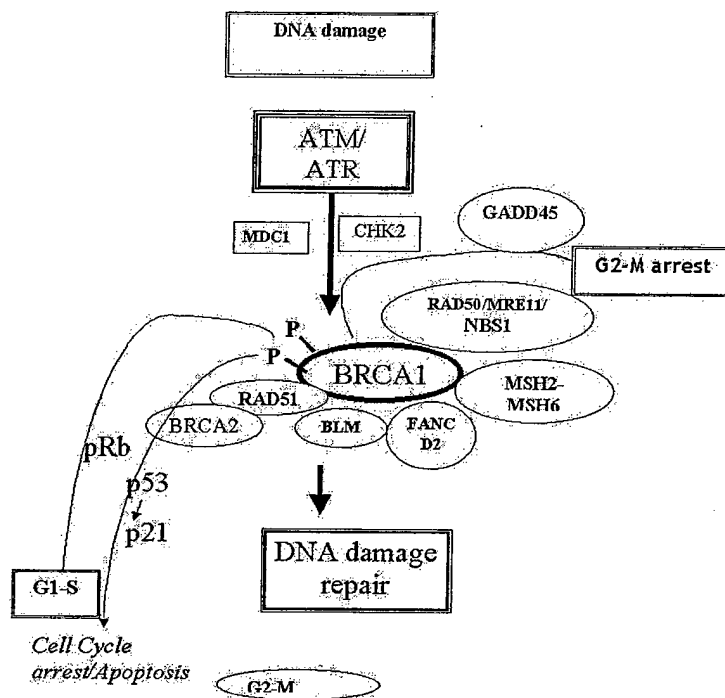
Evidence of a BRCA1/BARD1 interaction stems from immunofluorescence localization studies which show that both proteins co-localize to S-phase nuclear dots or foci (70). Neither BRCA1 nor BARD1 form nuclear foci in G1; however, they come together just before the S phase. Unlike BARD1, which shows constant levels throughout the cell cycle, the expression of BRCA1 is generally absent or low during G1, but it peaks before the S-phase. BARD1 and RAD51 localize to PCNA nodules following treatment with hydroxyurea or UV (71,72).

Double strand breaks (DSB) are considered to be highly dangerous lesions in cells, such breaks can be generated by various genotoxic agents, from exogenous

and endogenous sources. Exogenous sources include ionizing radiation (IR), radiomimetic agents, and chemotherapeutic agents. Endogenous agents are generated by mechanical stress and reactive oxygen species. In addition, endogenous topoisomerase, DNA cleavage, replication, meiosis, and fragile site formation can also generate DSB. Two distinct mechanisms have been established for the repair of DSB: I) Homologous recombination (HR) uses a sister chromosome as a template for repair; II) non-homologous end-joining (NHEJ), rejoins two broken ends of DNA directly.

One of the clues linking BRCA1 to DNA repair was its association with Rad51, the primary RecA homolog in eukaryotic cells (70,71,73). RAD51 shares significant homology with bacterial RecA, which has been shown to mediate the pairing and ATP-dependent exchange of DNA strands in recombination (74). RAD51 interacts with the C-terminal region of the BRCA1 protein, between amino acid 758 and 1064. The BRCA1 protein co-localizes with Rad51 in nuclear dots during the S-phase and in response to DNA damage, suggesting that it may also be involved in homologous recombination and recombinational repair. BRCA1 null mice suffer embryonic lethality and are very similar in phenotype to mice lacking Rad51 or BRCA2





**Figure 2.** A model showing interaction of BRCA1 with other proteins in response to DNA damage. In this case, BRCA1 is phosphorylated by ATM/ATR. BRCA1 forms complexes to repair Double Strand Breaks (DSB) by homologous recombination. BRCA1 also has a role in cell cycle arrest at G2-M checkpoint, possibly via upregulation of GADD45. The BRCA1-associated genome surveillance complex (BASC)-containing RAD50/MRE11/NBS1, DNA mismatch repair proteins, DNA helicase BLM (Bloom's syndrome protein) and others are in sensing and repair of abnormal DNA. BRCA1 may interact with p53 pathways to initiate cell cycle arrests/apoptosis, if necessary, or interacts with Rb to control G1-S stage of cell cycle (Ref. 50, 77,79).

genes. These embryos display cellular proliferation defects, are sensitive to ionizing radiation, and exhibit high levels of chromosomal abnormalities; the latter can be partially rescued by p53 mutation.

BRCA1 is associated with Rad50 as a part of the Mre11/Rad50/Nbs1 (nibrin) complex (MRN) (75,76), which is involved in both nonhomologous end joining (NHEJ) and homologous recombination in yeast and vertebrate cells (74,77,78,79). The MRN complex localizes to the sites of DSB *in vivo* and plays a critical role in DNA metabolism, including DSB repair, meiotic recombination, and telomere maintenance. Cells deficient in Mre11 or Nbs1 continue DNA replication following X-ray damage, and thus display defective checkpoint signaling during the S-phase. In response to double strand breaks BRCA1 is phosphorylated by ATM, a kinase that phosphorylates the multiple protein complex (75,80,81). ATM also phosphorylates Nbs1 in response to DNA damage (75,82,83). The BRCA1 foci that appear after ionizing radiation, are co-localized with the subset of foci formed by the MRN complex, re-stating a role for BRCA1 in the cellular response to DSB. In addition, BRCA1 is also involved in another kind of DNA repair, namely base excision repair (BER) (84). Embryonic stem cells of

BRCA1-deficient mice exhibit defects in transcription-coupled repair, which are generally the result of accumulation of oxidized bases following insult by ionizing radiation. BRCA1 may manifest its role in BER through its association with mismatch repair enzyme (85) or by transcription via its interaction with RNA polymerase II holoenzyme. During the S-phase of the mitotic cell cycle, BRCA1 colocalizes with RAD51 in subnuclear structures, Scully *et al.* (1997)(70,71), known as "BRCA1 nuclear dots" that are succulent in nature. However, in response to DNA damage these dots appear to disperse (70,71). BRCA1 interacts with BRCA2, which directly binds with RAD51 (77,79). Recently it has been shown that BRCA1 co-purified with several proteins that are associated with the DNA damage machinery, including mismatch repair enzyme, MSH2, MSH6, MLH1, ATM, BLM, as well as MRN protein complex, in a single complex referred as the BRCA1-Associated Surveillance Complex (BASC) (75). BASC contains at least 15 subunits and is involved first in sensing damaged DNA and then in repairing it. All of these BRCA1-associated proteins may have BRCA1-dependent as well as independent functions and BRCA1 may regulate such proteins for a specialized repair. Figure 2 illustrates that BRCA1 forms various complex(es) with DNA-repair proteins to participate in repair of damaged

DNA or accelerate cell cycle arrest. Also, DNA replication factor C, itself a protein complex that recruits PCNA onto DNA polymerase  $\delta$ , was found to be a component of BASC. Many proteins in BASC are tumor suppressors, indicating that loss of integrity of BASC may be a central mechanism in tumor development. The MSH mismatch proteins are involved in repair of mismatched DNA lesions is due to spontaneous errors during DNA replication or during repair of DSBs. It is possible that a MSH2-MSH6 complex may signal regulation of down-stream events, such as apoptosis or cell cycle arrest via BRCA1. In this manner, MSH2-MSH6 as a multi-protein complex interacts with the repair machinery and modulates cell cycle checkpoints and apoptosis decisions. The DNA helicase gene Bloom-syndrome (BLM) may be involved in sensing abnormal double strand DNA structures formed during replication). R/M/N protein complex is involved in DNA repair at stalled replication forks (87). Both ATR and ATM are components of BASC, which can phosphorylate numerous proteins of the BASC complex (89,90). BRCA1 can participate in DNA repair in many ways. One possibility is that BRCA1 acts as a scaffold protein. BRCA1 may also exert local activities at DSB sites via its interaction with enzymes that alter chromatin and DNA structures. BRCA1 interacts with SWI/SNF and other proteins that remodel chromatin with regulators of histone acetylation/deacetylation (reviewed by 77,78,79). BRCA1 also interacts with DNA helicases, including the RecQ homolog encoded by Bloom's syndrome gene, BLM and the helicase BACH1 (88). Perhaps these interactions are required for the accessibility of the repair machinery. One response of BRCA1 to DNA damage is to monoubiquitinate histones (H2A and H2B) in conjunction with BARD1; in this way chromatin remodeling takes place that could allow the DNA repair machinery to gain access to the damaged DNA.

The two kinases, CHK1 and CHK2 are responsible for the maintenance of the G2-M DNA damage checkpoint. CHK2 phosphorylates BRCA1 in response to DNA damage induced by IR. Chk1 and Chk2 are classic serine-threonine kinases that are required for cell cycle arrest in response to DNA damage. As downstream kinases, they are phosphorylated by an ATM/ATR-dependent process and then Chk2 phosphorylates BRCA1 (87,89). BRCA1 phosphorylates CHK1 to control G2-M transition. Reciprocal co-immunoprecipitation of BRCA1 and CHK1 has been shown in HeLa and MCF7 cells. In BRCA1 mutant HCC1937 cells, where BRCA1 is expressed, colocalization of BRCA1 and CHK1 is maintained even after gamma-radiation (90). It has been proposed that, in response to DNA damage, BRCA1 controls cell cycle progression to mitosis via CHK1, which regulates Cdc2 kinase, Cdc25C and WEE1 (91). Some studies suggest that BRCA1 forms a complex with the transcriptional co-repressor complex CtP and CtBP through its BRCT domain, and during the DNA damage response, this complex gets dissociated from BRCA1, which, in turn, activates GADD45 and p21.

Specific sites in BRCA1 are responsive to DNA damage repair stimuli. BRCA1 becomes phosphorylated in

response to treatment of cells with a variety of DNA damaging agents, such as, UV, IR, adriamycin, hydroxyurea, mitomycin C, and hydrogen peroxide. Multiple phosphorylation sites at the serine (S) residue, including S1330, S1423, S1466, S1466, S1524 and S1542, have been detected by mass spectrometry analysis of recombinant BRCA1 peptides, phosphorylated *in vivo* in an ATM-dependent manner (89). These phosphorylations may lead to a change in BRCA1 subnuclear localization. Among specific BRCA1 phosphorylation sites that have been responsive to DNA damage. For example, in MCF-7 cells, IR- and UV-induced phosphorylation of BRCA1 at Ser-988/-1524 and Ser-988, respectively, was seen during the S-phase (92); however, in the G2/M phase, IR and UV treatment induced phosphorylation of Ser-988/ser-1423 and Ser-1423, respectively (92). In HCC1937 cells, with specific BRCA1 mutation where the functional C-terminal BRCT domain is lost, phosphorylation of Ser-1423 and -1524 was not induced. It is possible that allosteric change of the BRCA1 structure due to phosphorylation, may affect its interaction with other proteins involved in DNA damage repair pathways (93).

## 3. CONCLUSIONS

Thus, in summary, it is evident from the discussion presented above that BRCA1 serves as one of the important tumor suppressor genes in the etiology of the breast cancer, particularly in high risk families. The wide spectrum of mutations observed in the gene in various populations of the world, with a few exceptions, is not specific to any particular population and mutations are scattered throughout the coding region of the gene. While BRCA1 follows Knudson's "two hit" hypothesis, in familial early on set cancers, the mode of its inactivation, in the much more common in sporadic cancers, is poorly understood; the promoter hypermethylation as a mechanism of inactivation is prevalent only in less than half of the cases. A significant amount of work, therefore, is needed to elucidate the role of BRCA1 in sporadic cancers. Its physiological role in DNA damage sensing and repair sheds light on its function as a caretaker to maintain the genomic stability. Inactivation of BRCA1 confer on cells, new genetic abnormalities; this, in turn, leads to tumorigenesis. Future studies will focus on elucidating the mechanisms of BRCA1 in the multistep process of tumorigenesis relating to sporadic cancers, and eventually means to prevent cancers.

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Involvement of p53 and BRCA1 genes in Breast Cancer in African-American and White Women

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